이학박사 학위논문

# Studies on the Initiation of DNA Replication Induced by RecQL4 Tethering on the Pre-Replicative Complex

## RecQL4의 복제전복합체 결합에 의해 유도되는 DNA 복제의 개시 연구

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# ABSTRACT

# Studies on the Initiation of DNA Replication Induced by RecQL4 Tethering on the Pre-Replicative Complex

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The mechanism of origin activation in eukaryotic DNA replication has been studied extensively in yeast systems. While mammalian homologues of yeast proteins, such as RecQL4, appear to play conserved roles, detailed mechanisms of origin activation, and its control, are still poorly understood in mammalian cells. RecQL4, a member of the conserved RecQ family helicases, plays an essential role in the initiation of DNA replication in mammalian cells. To analyze the molecular interaction and function of proteins required for origin activation in mammalian cells, the RecQL4 protein was tethered to the pre-replicative complex (pre-RC) by expressing RecQL4-Orc4 fusion proteins in HeLa cells; the recruitment of other initiation

factors and occurrence of origin firing was then observed in late replicating origins. RecQL4 protein tethered on the pre-replicative complex induces early activation of late replicating origins during S phase. Tethering of RecQL4 or its N-terminus on pre-RCs resulted in the recruitment of essential initiation factors, such as Mcm10, And-1, Cdc45, and GINS, and this increased nascent DNA synthesis in late replicating origins during early S phase. This early activation of late replicating origins induced by RecQL4-Orc4 requires both Mcm10 and And-1. In addition, tethered RecQL4 was able to recruit Cdc45 even in the absence of cyclin-dependent kinase (CDK) activity, and CDK phosphorylation of RecQL4 N-terminus was required for interaction with and origin recruitment of And-1 and GINS. Furthermore, forced activation of replication origins by RecQL4 tethering resulted in increased replication stress and the accumulation of single stranded DNAs, which can be recovered by transcription inhibition. Collectively, recruitment of RecQL4 on replication origins appears to be an important step for temporal activation of replication origins during S phase. Further, perturbation of replication timing control by unscheduled origin activation significantly induces replication stress, which is mostly caused by transcription-replication conflicts. This study provides significance of RecQL4 in replication origin activation and a useful model system for studying replication initiation and replication stress.

**Key words** : RecQL4, CDK, replication initiation, origin activation, replication stress, transcription-replication conflicts, replication timing control

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# TABLE OF CONTENTS

# List of Figures

# Abbreviations

| | |
|---|---|
| A1 | And-1 |
| ATM | ataxia telangiectasia mutated |
| ATR | ataxia telangiectasia and rad3-related protein |
| Bl | bleomycin |
| BrdU-IP | bromodeoxyuridine-immunoprecipitation |
| CDK | cyclin-dependent kinase |
| CMG | Cdc45-Mcm2-7-GINS |
| Co | cordycepin |
| DAPI | 4'-6'-diamidino-2-phenylindole |
| DDK | dbf4-dependent kinase |
| dNTP | deoxynucleotide |
| DSB | double strand break |
| Eto | etoposide |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GINS | go-ichi-ni-san, Sld5, Psf1, Psf2 and Psf3 complex |
| GRM8 | metabotropic glutamate receptor 8 isoform |
| γH2AX | phosphorylated histone H2AX |
| kDa | kilo dalton |
| Mcm | minichromosome maintenance |
| M10 | Mcm10 |
| M-O4 | Mcm10-Orc4 fusion protein |
| Nu | Nu6140 |
| Nuc | nucleosides |
| ORC | origin recognition complex |

| | |
|---|---|
| O3-A | Orc3-And-1 fusion protein |
| O4 | Orc4 |
| PHA | PHA-767491 |
| PP1 | protein phosphatase 1 |
| pre-RC | pre-replicative complex |
| qPCR | quantitative polymerase chain reaction |
| R4 | RecQL4 |
| R-O4 | RecQL4-Orc4 fusion protein |
| Ros | Roscovitine |
| R-loops | DNA:RNA hybrids |
| RPA | replication protein A |
| siRNA | small interfering RNA |
| TRCs | transfcription-replication conflicts |

# Ⅰ. Background

## 1.1. Initiation of eukaryotic DNA replication

### 1.1.1. Replication origin

Precise control of the initiation of DNA replication is important for faithful duplication of the genome in eukaryotes, which contains multiple replication origins.

In *Saccharomyces cerevisiae*, replication starts from specific short sequences of the genome known as autonomous replication sequences (ARSs). ARS elements, although diverse, maintain a basic structure composed of three domains: A, B, and C. Domain A contains the consensus sequence, [(A/T)TTTAT(A/G)TTT(A/T)], designated the ARS consensus sequence (ACS); domain B contains a DNA unwinding element and domain C is important for DNA-protein interaction (Dhar et al., 2012).

In contrast, evidence regarding consensus sequences has not been established in *Saccharomyces pombe*. Instead of consensus sequences, *S. pombe* origins feature AT-rich elements (Cotobal et al., 2010; Maundrell et al., 1988). In mammalian cells, the location and distribution of replication origins throughout the genome define replicons large sequence domains copied by the bidirectional movement of the replication fork away from an origin (Prioleau and MacAlpine, 2016). For

example, lamin B2 origin spans approximately 1 kb and the human β-globin gene locus, whose length is 40 kb, contains several replication origins (Kamath and Leffak, 2001). Moreover, mammalian replication origins correlate with DNA structures such as CpG island regions (Delgado et al., 1998) and G-quadruplexes (Prorok et al., 2019). Additionally, epigenetic features are associated with origins. H4K20 methylation has been reported to be required for efficient replication initiation. The epigenetic modification of the Orc1-BAH (bromo-adjacent homology) domain facilitates ORC association with chromosomes and Epstein-Barr virus origin-dependent replication (Ekundayo and Bleichert, 2019).

### 1.1.2. Pre-replicative complexe (pre-RC) formation

For complete duplication of the whole genome once in a cell cycle, pre-replicative complexes are assembled on replication origins in G1 phase and sequentially activated during S phase in eukaryotes (Parker et al., 2017). Origin recognition complexes (ORC) bind to replication origins after mitosis. Next, Cdc6, Cdt1, and the minichromosome maintenance2-7 (Mcm2-7) are recruited to form the pre-RC in G1 phase.

Eukaryotic ORC recognizes DNA in an ATP-dependent manner (Bell and Stillman, 1992). Five of the six subunits of ORC (Orc1, Orc2, Orc3, Orc4, and Orc5) retain AAA+ modules, followed by a single C-terminal WH domain (winged-helix DNA-binding domain); the sixth (Orc6) is composed of tandem

cyclin-box folds similar to those observed in transcription factor IIB (TFIIB). The AAA+ subunits are arranged in the order of Orc1 - Orc4 - Orc5 - Orc3 - Orc2. To load the ORC onto DNA, the Orc6 C-terminal domain (CTD) binds to Orc3 and the TFIIB domain binds DNA elements. The Orc1 AAA+ domain repositions to interact with Orc4, then the Orc2 WH domain is relocated thus, binding it around the DNA. Finally, the DNA is then bound to the central channel of ORC (Bleichert et al., 2015).

Cdc6 is another AAA+ ATPase. Cdc6 binds between Orc2 and Orc1, trapping DNA inside the complex; this requires ATP. The ORC/Cdc6 complex is essential for the recruitment of the Cdt1/minichromosome maintenance 2 - 7 (Mcm2 - 7) heptamer, a major component of eukaryotic DNA helicase, and the loading of Mcm2 - 7 on dsDNA (Bleichert et al., 2015; Riera et al., 2017). Cdt1-Mcm2-7 assembles into a ring structure immediately adjacent to ORC-Cdc6 (Zhai et al., 2017), creating the ORC/Cdc6/Cdt1/Mcm2 - 7 (OCCM) complex (Evrin et al., 2013). Following Cdc6 and Cdt1 release by ATP hydrolysis (Ticau et al., 2015), another Cdc6 is loaded onto ORC/Mcm2-7 creating the ORC/Cdc6/Mcm2 - 7 (OCM) complex (Fernández-Cid et al., 2013). Then, a Cdt1-Mcm2-7 complex is added to the OCM complex, leading to the formation of an Mcm2-7 double hexamer (DH) (Fernández-Cid et al., 2013; Ticau et al., 2015). Finally, Cdc6 and Cdt1 are released from the DH, forming a stable complex (Ticau et al., 2017).

### 1.1.3. CMG complex formation and its control by CDK and DDK

For the onset and progression of S phase, pre-RCs on individual replication origins are sequentially activated by forming the active replicative helicase complex containing Cdc45, Mcm2-7, and GINS, which is called the CMG complex. In yeast systems, this activation process requires many origin firing factors, including Dpb11, Sld2, Sld3, and Mcm10, and two S phase promoting kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK) (Muramatsu et al., 2010; Tanaka et al., 2011). DDK phosphorylates Mcm4 and Mcm6, leading to recruitment of Cdc45 and Sld3 (Deegan et al., 2016; Duncker, 2017; Masai et al., 2006). CDK phosphorylates Cdc45, Sld2, Sld3, and Sld7, and together with Dpb11 it facilitates recruitment of a protein complex composed of GINS, Sld2, and Pol ε which is responsible for leading strand synthesis. In other words, Sld2 and Sld3 are phosphorylated by CDK, leading to simultaneous binding with Dpb11 (Muramatsu et al., 2010; Tanaka et al., 2007). Association of these proteins together with Mcm10 and the action of these two kinases trigger assembly and activation of the CMG complex (Heller et al., 2011; Parker et al., 2017; Yeeles et al., 2015). In summary, Cdc45 has been suggested to be recruited in the form of a Sld3 - Cdc45 complex, while GINS has been suggested to be recruited via Sld2 (Reusswig and Pfander, 2019).

## 1.2 Pre-RC formation and CMG assembly in human DNA replication initiation

The process of pre-RC formation and origin activation appears to be fairly conserved in all eukaryotic systems and human homologues of yeast proteins play similar roles during the initiation process. However, the two processes differ in their detail. The pre-RC forms at the replication origin whereas Mcm2-7 loading requires other factors. GRWD1 (glutamate-rich WD40 repeat containing 1) binds to two representative replication origins specifically during the G1 phase in a Cdc6- and Cdt1-dependent manner; depletion of GRWD1 reduces loading of Mcm2-7, but not Cdc6 and Cdt1, suggesting that GRWD1 might relax histone-DNA interactions to increase the ratio of nucleosome-free DNA rather than simply displacing histones (Sugimoto et al., 2015). HBO1 (human acetylase binding to Orc1; also known as KAT7 and MYST2; H4-specific histone acetylase) interacts with Mcm2 and Orc1(Burke et al., 2001; Iizuka and Stillman, 1999) and its enzymatic activity is essential for Mcm2-7 loading (Miotto and Struhl, 2010). SNF2H (Sucrose Nonfermenting 2 Homolog; a ATP-dependent chromatin remodeler) is recruited onto DNA replication origins in human cells in a Cdt1-dependent manner and is required for efficient Mcm2-7 loading (Sugimoto et al., 2011).

For Cdc45 loading onto the pre-RC, human cells require Treslin, a Sld3 candidate homologue. Treslin and TopBP1 (DNA topoisomerase 2-binding protein 1) interact with Cdc45 (Kumagai et al., 2011; Schmidt et al., 2007) and are required for Cdc45 recruitment to chromatin (Kumagai et al., 2010). Treslin is phosphorylated by cyclin A/CDK2 and promotes the Treslin-TopBP1 interaction and Cdc45 loading (Boos et al., 2011; Kumagai et al., 2011). Also, MTBP (MDM2 binding protein) interacts with Treslin throughout the cell cycle and MTBP depletion inhibits DNA replication by preventing assembly of the CMG helicase during origin firing (Boos et al., 2013). On the other hand, TopBP1 is dispensable for CMG assembly as observed in bimolecular fluorescence complementation (BiFC) analysis (Im et al., 2009). Therefore, the role of TopBP1 in CMG activation is controversial. In addition, the DUE-B (DNA unwinding element-binding protein) C-terminal domain is required to interact with Treslin and this interaction is essential for the loading of Treslin, DUE-B, and Cdc45 onto chromatin (Poudel et al., 2018). Moreover, DUE-B's C-terminal phosphorylation by DDK is important for Cdc45 chromatin association (Gao et al., 2014).

As for GINS loading onto the pre-RC, the binding of Mcm2 and Cdc45 is required; Cdc45 loading requires Sld5 (Im et al., 2009). And-1, which forms a complex with DNA

polymerase α (Zhu et al., 2007), interacts with GINS by Cdc7 phosphorylation of Sld5 (Tatiana et al., 2017). In addition, Mcm10 and And-1 are required for GINS interaction with Mcm2-7 and Cdc45 (Im et al., 2009). DNA polymerase α is responsible for synthesizing short RNA primers to produce nascent DNA strands. Additionally, DNA polymerase epsilon (pol ε) is required to recruit GINS to the MCM helicase in yeast (Muramatsu et al., 2010), but there is no evidence that this is the case in human cells (Moiseeva and Bakkenist, 2018).

## 1.3. Roles of RecQL4 in DNA metabolism

RecQL4 is a member of conserved RecQ family helicases that play important roles in the maintenance of genome integrity by acting in various DNA metabolic processes, such as DNA repair, DNA recombination, and DNA replication. In humans, five RecQ helicases, RecQL1, WRN, BLM, RecQL4, and RecQL5 have been identified. Mutations in WRN, BLM, and RecQL4 genes have been shown to be associated with human genetic disorders such as Werner, Bloom, and Rothmund-Thomson syndromes, which are characterized by increased genome instability, resulting in early aging symptoms and cancer predisposition (Larizza et al., 2006; Monnat Jr, 2010; Singh et al., 2012). While all these proteins contain a well-conserved helicase domain and contribute to genome maintenance, the roles of each protein in DNA metabolism are very different.

RecQL4 is known to be involved in many DNA metabolic processes. RecQL4 plays important roles in DNA double strand break (DSB) repair, such as homologous recombination and non-homologous end joining (Lu et al., 2017; Lu et al., 2016; Shamanna et al., 2014), and has also been shown to be involved in the activation of ataxia telangiectasia mutated (ATM), a major checkpoint kinase against DNA DSBs (Park et al., 2019). RecQL4 has a helicase domain, which shows 3' to 5' helicase activity (Xu and Liu, 2009). The conserved

helicase domain in RecQL4 and its helicase activity are required for these cellular responses to DNA DSBs (Lu et al., 2016; Park et al., 2019). Also, RecQL4 participates in telomere maintenance by assisting in telomeric D-loop resolution (Ghosh et al., 2012). In addition, RecQL4 has a unique N-terminal domain showing limited homology to Sld2, which is an essential replication initiation factor in yeast cells (Muramatsu et al., 2010), and this N-terminal domain has been shown to be essential for the initiation of DNA replication in vertebrates (Abe et al., 2011; Sangrithi et al., 2005).

## 1.4. Replication stress and cellular responses

Replication stress is defined as the slowing or stalling of replication fork progression (Mazouzi et al., 2014). For maintaining genome stability, cells should properly relieve replication stress, which might cause DNA damage if it is not relieved. Once cells in S phase are treated with chemicals such as hydroxyurea (HU), aphidicolin or etoposide, a mechanism called replication checkpoint pathway is triggered to prevent replication fork collapse.

Interruption of replication fork progression generate single stranded DNA (ssDNA) coated by Replication Protein A (RPA). ATR (ATM and Rad3 related) and ATRIP (ATR-interacting protein) are recruited onto RPA-coated ssDNA (Cortez et al., 2001; Zou and Elledge, 2003). Then, RAD17-replication factor C loads RAD9 - RAD1 - HUS1 (9-1-1) complex on DNA (Bermudez et al., 2003; Zou et al., 2003). The 9-1-1 complex is required for TopBP1 loading and function (Delacroix et al., 2007; Lee et al., 2007). TopBP1 interacts with ATR through ATRIP, and C-terminal domain of TopBP1 is responsible for ATR activation (Akiko Kumagai et al., 2006). Eventually, ATR activates its downstream kinase CHK1, resulting in blocking of origin firing and cell cycle arrest (Saldivar et al., 2017).

In addition to external reasons, spontaneous replication

stress arises. Common Fragile Sites (CFSs) are specific chromosomal loci that are prone to forming visible gaps and breaks on metaphase chromosomes (Glover et al., 2017). CFSs have AT-rich sequences that are difficult to be replicated (Arlt et al., 2002; Ried et al., 2000). They are also in large genes such as CFSs FRA3B in 1.5Mb FHIT gene and FRA16D in 1.1 Mb WWOX gene (Bednarek et al., 2000; Ohta et al., 1996). CFSs are also replicated in late S phase (Le Beau et al., 1998). These characteristics make cells to undergo replication stress.

In S phase, replication and transcription occasionally happen in the same area at the same time. As a result, transcription-replication conflicts (TRCs) arise. It is an encounter between replication and transcription machinery. There are two types of the conflicts, co-directional and head-on oriented TRCs (Hamperl et al., 2017). It features the formation and accumulation of R-loops. An R loop is a three-strand nucleic acid structure formed by an RNA:DNA hybrid plus a displaced DNA strand (ssDNA) (Aguilera and García-Muse, 2012). TRCs can cause DNA damage leading to replication stress and genomic instability—a hallmark of cancer. (Gaillard et al., 2015; García-Muse and Aguilera, 2016).

# Ⅱ. Introduction

For accurate replication of the genome, the process of replication origin activation is precisely programmed. In a mammalian cell, 30,000-50,000 replication origins are activated at specific times during the S phase of DNA replication (Mechali, 2010). Other origins that are not normally activated (dormant origins) can be activated when replication stress occurs (Blow et al., 2011). Replication origins are divided into three categories according to timing: early-, middle-, and late-firing origins (Aladjem, 2007). In contrast, the conversion of activation origins into replication zones occurs according to cell type. For example, the human β-globin origin region is replicated early during S phase in erythroid cells but late during S phase in non-erythroid cells (Goren et al., 2008; Kitsberg et al., 1993).

RecQL4, the human homologue of yeast Sld2, plays essential roles in the assembly of the CMG complex and origin activation. RecQL4 interacts with other essential initiation factors, such as Mcm10 and And-1/Ctf4/Wdhd1, and is required for origin association of these proteins and components of the CMG complex, Cdc45 and GINS (Im et al., 2015; Xu et al., 2009). Origin association of RecQL4 and interaction of RecQL4 with Mcm10 and And-1 on replication origins depend on CDK and DDK (Im et al., 2015). And CDK phosphorylation of the RecQL4 N-terminus has been shown to reduce the physical

interaction with Mcm10 in a salt-dependent manner *in vitro* (Xu et al., 2009). However, roles of RecQL4 and other initiation proteins leading to origin activation and control of this process by CDK and DDK during S phase are not well understood in mammalian cells. Human CMG activation requires MCM phosphorylation by DDK (Cho et al., 2006; Tsuji et al., 2006). As And-1 is required for Mcm4 hyperphosphorylation by DDK, an unscheduled replication initiation marker, its C-terminus interacts with Psf3 (Moiseeva et al., 2017). Given that the direct interaction between And-1 and DDK remains elusive, and that And-1 origin binding depends on RecQL4 (Im et al., 2015), it is possible that RecQL4 (and its modifications) might control the molecular mechanisms between And-1 and GINS for origin activation. Although CDK regulates the interaction among RecQL4, Mcm10, and And-1, as well as their origin association for origin activation, CDK phosphorylation targets on RecQL4 and their functions remain unclear. In addition, since RecQL4 is loaded onto chromatin beginning early in the G1 phase (Thangavel et al., 2010), it is likely to play a role in specifying the origin to be activated.

Eukaryotic DNA replication initiation has been well studied in yeast. In addition assay systems for DNA replication initiation have been established. For instance, DNA replication was reconstituted *in vitro* (Yeeles et al., 2015). On the other hand, currently, there is no DNA replication initiation assay system in use in human cells. The protein depletion technique

using small interfering RNA (siRNA) is a powerful tool for studying the function of a gene. Although the technique is extensively used to investigate mechanisms of DNA replication initiation, also there is inadequacy exist. The knockdown of replication-related proteins inhibits DNA replication and disrupts the normal cell cycle. For example, the depletion of Cdc7 or Sld5, key components of DNA replication, causes cells to remain in the G1 phase and delays the progression of the S phase (Aparicio et al., 2009; Montagnoli et al., 2004), making it difficult to investigate what is occurring during this period. In addition, the bimolecular fluorescence complementation (BiFC) assay is useful for detecting molecular interactions between two adjacent proteins, but this is not suitable for observing the resulting nascent DNA synthesis created by the interaction (Im et al., 2009). In addition, both assays do not reflect what is occurring at the replication origin, in particular in elucidating how replication proteins function in origin activation. Therefore, a new system is required to compensate for the inability of either assay to fully investigate the mechanism of DNA replication initiation.

In this study, I forced the binding of wild-type or CDK phospho-mutant RecQL4 proteins to replication origins by expressing RecQL4 proteins fused to Orc4, a component of ORC, in HeLa cells. I then examined the recruitment of initiation factors on replication origins and the time of origin

activation during S phase. I found that the tethered RecQL4 on the pre-RC led to early activation of late replicating origins and increased replication stress, which was alleviated by transcription inhibition. Conversely, phospho-deficient RecQL4 protein for CDK phosphorylation failed to induce early activation of late origins due to a lack of And-1 and GINS binding. My results indicate that recruitment of RecQL4 on replication origins is an important step for temporal activation of replication origins during S phase. Furthermore, these results provide an insight for CDK control of origin activation and replication timing control during S phase in mammalian cells.

# Ⅲ. Materials and Methods

## 3.1. Cell culture and synchronization

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene, Korea) and antibiotics (Welgene, Korea). HeLa or U2OS cells stably expressing RecQL4-Orc4 from a doxycycline inducible promoter (HeLa-RO4 or U2OS-RO4) were cultured in DMEM supplemented with 10% tetracycline-free FBS (Takara) and antibiotics. Cells were maintained in 5% $CO_2$ humidified incubator at 37 ℃.

To synchronize the cell cycle, cells were treated with 2 mM thymidine (Sigma) for 18 h, washed with Phosphate-Buffered Saline (PBS; Welgene, Korea) 2 times, grown in fresh medium for 12 h, and further incubated for 13 h in medium containing 2 mM thymidine.

## 3.2. Cell cycle analysis

For monitoring cell cycle, cells were harvested by trypsinazation, washed with PBS and fixed with 70% ethanol in PBS at -20℃. After ethanol was removed, cells were resuspended in PBS with RNase A (2 μg/ml) and incubated for 30 min. Then, propidium iodide (10 μg/ml) was treated for 10 min. Cell cycle profiles were observed by flow cytometry analysis using FACS Calibur (BD Biosciences, USA).

For ectopic protein expression in synchronized cells, transfection was carried out at the time of release from the first thymidine block.

## 3.3. Plasmids preparation

For wild-type or various mutant RecQL4 Orc4 fusion protein expression, wild-type RecQL4 cDNA (amino acid residues 1-1209), C-terminal region cDNA (1-241 for CD1 and 1-427 for CD2), and N-terminal cDNA (ND, 248-1209) were amplified by Polymerase Chain Reaction (PCR) and inserted into the N-terminus of the ORC4 gene, which was cloned into the pcDNA3.1 (-) plasmid.

Wild-type Mcm10, wild-type And-1, Mcm10 fused to the N-terminus of Orc4, and Orc3 fused to the N-terminus of And-1 were also generated by PCR and subcloned into the pcDNA3.1 (-) plasmid.

To generate a HeLa or U2OS cell line stably expressing RecQL4-Orc4 protein from a doxycycline-inducible promoter, the RecQL4-Orc4 expression vector was constructed using the pTRE plasmid.

## 3.4. Site-directed mutagenesis

For alanine or glutamic acid substitution of the CDK phosphorylation sites of RecQL4 (S89, T93 and T139), site-directed mutagenesis was conducted using PCR (Ho et al.,

1989; Reikofski and Tao, 1992). The front and rear fragment for each target site were amplified using primers with designated mutation. The former and latter segments were mixed to 1:1 molar ratio, and PCR was performed again to make a complete fragment. Then, the mutated RecQL4 cDNA was sub-cloned into Orc4 pcDNA 3.1 (-) plasmid.

## 3.5. Stable cell line construction

RecQL4-Orc4 pTRE plasmids were transfected into HeLa or U2OS Tet-On cells. Cells were treated with hygromycin (150 μg/ml) for 2 weeks with medium exchange every 2 days. Subsequently, a cell line stably expressing the RecQL4-Orc4 fusion protein was screened by Western blot analysis.

## 3.6. Plasmids and small interfering RNA (siRNA) transfection

Transfection of plasmid DNAs was performed with Polyfect (Qiagen, Germany), and transfection of siRNAs were performed using the Neon transfection system or Lipofectamine 3000 (ThermoFisher Scientific, USA) following the manufacturer's instruction. All siRNAs were chemically synthesized from Bioneer (Daejeon, Korea), and sense-strand sequences of siRNAs used in this study were as follows:

GL-2, 5'-AACGUACGCGGAAUACUUCGA-3';

RecQL4, 5'-GACUGAGGACCUGGGCAAA-3';

Mcm10, 5'-AGAGUUGCAAGAGGAAUUA-3';
And-1, 5'-GAUCAGACAUGUGCUAUUA-3'.

## 3.7. Western blot analysis

For western blot analysis, cells were re-suspended in a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 200 μM sodium orthovanadate, and protease inhibitors). The cells were disrupted by sonication, and 20 μg of proteins was subjected to SDS polyacrylamide gel electrophoresis. The protein concentration in the lysate was measured by Bradford assay. Anti-RecQL4 antibody (Abfrontier, Korea) was prepared by immunizing rabbits with recombinant proteins of the RecQL4 N-terminus (amino acid residues 1-241). Anti-Orc2, anti-WDHD1/And-1, anti-phospho RPA32 (S33), and anti-γH2AX were purchased from Bethyl Laboratories (USA). Anti-Mcm4, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-Orc4, and anti-ATR antibodies were from Santa Cruz Biotechnology (USA), and anti-Lamin B1 and anti-Mcm10 antibodies from Abcam (UK). Anti-HA, anti-phospho ATR (S428), and anti-Cdc45 antibodies were from Cell Signaling Technology (USA). Anti-GINS4 (Sld5) was from GeneTex (USA). Anti-FLAG, anti-RPA32, and anti-BrdU antibodies were supplied by Sigma-Aldrich (USA).

## 3.8. Co-Immunoprecipitation

To prepare whole cell extracts for IP, cells were lysed in a buffer (25 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM DTT, 0.2% IGEPAL CA-630, 10 mM sodium fluoride, 200 μM sodium orthovanadate, and protease inhibitors) containing 150 mM NaCl or 150 mM Na-acetate. Cell lysates were sonicated, treated with 0.25 U/μL benzonase (Enzynomics, Korea) at 4℃ for 1 h, and cleared by centrifugation at 13,000 rpm for 10 min. To block non-specific protein binding, PBS rinsed anti-FLAG beads (Sigma-aldrich, USA) were incubated in washing buffer (25 mM Tris-HCl, pH 7.5, 150 mM Na-acetate or 150 mM NaCl, 2.5 mM $MgCl_2$, 1mM DTT, 0.05% IGEPAL CA-630, 10mM sodium fluoride, 200 μM sodium orthovanadate and protease inhibitors) containing 3% BSA for 30min at 4℃. To pull down the target proteins, 200-400μg aliquots of the supernatant was incubated with the blocked anti-FLAG beads at 4℃ with gentle shaking. The beads were washed 4 times with washing buffer. After washing steps, 1X SDS-PAGE sample buffer was added to the beads and boiled.

## 3.9. Immunocytochemistry

For immunostaining, cells were grown on coverslips and pre-extracted in CSK buffer (10 mM PIPES pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 0.5% TritonX-100) on ice for 5 min. Subsequently, cells were fixed with 3.7%

paraformaldehyde, washed with PBS containing 0.25% Triton X-100, and incubated in a blocking buffer (PBS containing 5% bovine serum albumin and 0.1% Tween-20). Primary and fluorescent-conjugated secondary antibodies were sequentially treated in the blocking buffer for 1 h. Cell nuclei were stained with 0.2 μg/mL 4'-6'-diamidino-2-phenylindole (DAPI) in PBS for 3 min. The images of stained cells were obtained by fluorescent microscopy.

## 3.10. BrdU-IP, ChIP, and quantitative PCR analysis

BrdU-IP assays were performed to measure nascent DNA synthesis at replication origins as described previously (Azuara, 2006) with some modifications. Cells were pulse-labeled for 30 min with BrdU (150 μM) and harvested. Genomic DNA was extracted and sonicated to the average size of about 300 nucleotide pairs. After DNA denaturation by boiling and rapid cooling, the nascent DNA was immunoprecipitated with anti-BrdU antibody and Dynabeads protein G (ThermoFisher Scientific, USA) in an immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% bovine serum albumin, 0.1% Triton X-100). The beads were washed four times with washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and once with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA bound to the beads was eluted by 100 mM

Glycine-HCl (pH 1.9) and immediately neutralized by adding Tris-HCl buffer (pH 8.5).

ChIP assays were performed as described (J. S. Im et al., 2015), except for using Dynabeads protein G. Primer sets for the origin in Lamin B2 locus (LB2 Ori), metabotropic glutamate receptor 8 isoform locus (GRM8 Ori), β-globin locus (β-globin Ori), and the -21kb distal region of β-globin Ori (β-globin -21kb) were as follows:

LB2 Ori-F,5'-AATAAACTCAGAGGCAGAACC-3';
LB2 Ori-R, 5'-AGAAGATGCATGCCTAATGTG-3';
GRM8 Ori-F, 5'-GGGAAGGAAATGCAAGACAA-3';
GRM8 Ori-R, 5'-AATTTGGCTGCTTAGCATGG-3';
β-globin Ori-F, 5'-CTATTGCTTACATTTGCTTCTG-3';
β-globin Ori-R, 5'-CTTCATCCACGTTCACCTTG-3';
β-globin -21 kb-F, 5'-TGGCCACCAATTGAGTCATC-3';
β-globin -21 kb-R, 5'-AGTCAGGCTGGAAATGAGAG-3'.

Precipitated DNAs were analyzed by quantitative real-time PCR analysis using the Rotor-gene Q real-time PCR cycler (Qiagen, Germany). The percent enrichment of specific origin DNAs in BrdU-IP compared to input DNA was calculated for nascent DNA synthesis, and enrichment of β-globin origin DNA compared to the 21 kb distal region DNA was calculated for ChIP analyses. Data represent the mean value ± standard deviation for more than three independent experiments. Statistical significance was assessed using the two-tailed unpaired Student's t-test.

# Ⅳ. Results

## 4.1. Recruitment of RecQL4, Mcm10, and And-1 on late replicating origins occurs in late S phase

Activation of the replicative helicase on individual replication origins should occur at the time of origin activation. Since replication initiation factors such as RecQL4, Mcm10, and And-1 are required for CMG assembly in mammalian cells, I investigated when these factors were recruited to late replicating origins in S phase. To do this, HeLa cells were synchronized at early or late S phase using double thymidine block and release (**Figure 1A**), and the binding of these proteins to the replication origin in the β-globin locus (β-globin origin), a well-characterized late replicating origin in HeLa cells (Goren et al., 2008; Kitsberg et al., 1993), was examined by *in vivo* crosslinking followed by chromatin immunoprecipitation (ChIP). As shown in **Figure 1B**, binding of the pre-RC component proteins, Orc2 and Mcm4, was observed in both early S (released for 1 h from double thymidine block) and late S (released for 6 h) phases, while loading of Cdc45 and Sld5, components of the CMG complex, was very low in early S phase and significantly increased in late S phase. Binding pattern of the RecQL4, Mcm10, and And-1 proteins to the β-globin origin was similar to that of the CMG component proteins (**Figure 1B**), indicating that these proteins were

recruited to the late replicating origin only at late S phase, but not early S phase. These results suggest that their binding to individual replication origins is possibly regulated by the replication timing control program in cells, and might be an important control step to activate late replicating origins.

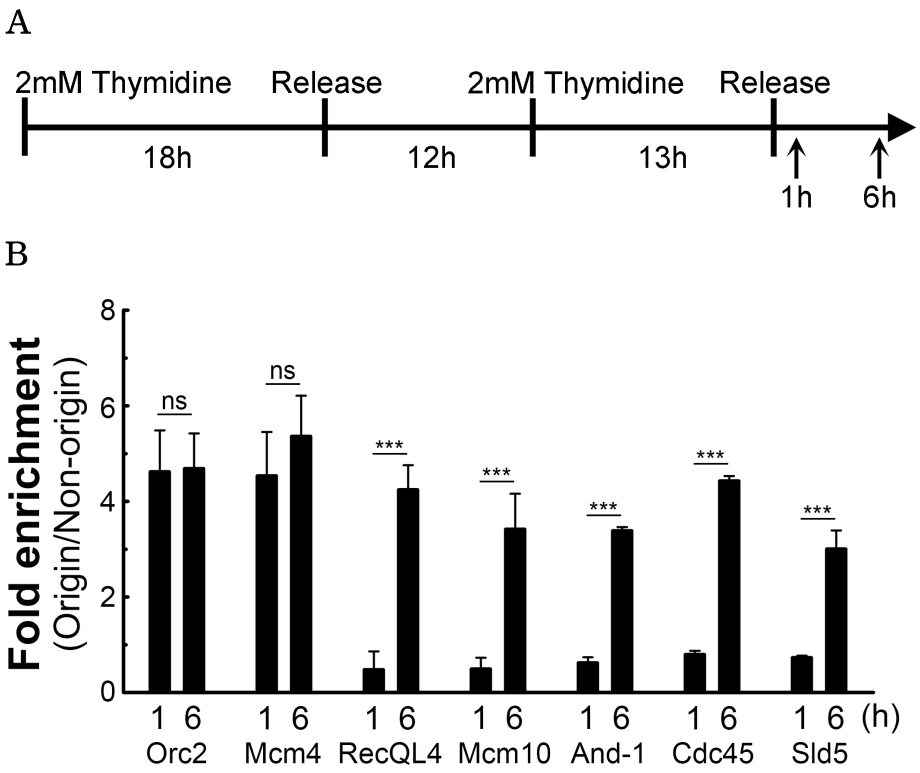



**Figure 1. Recruitment of replication factors for origin activation at the late replicating origin occurs in late S phase.**

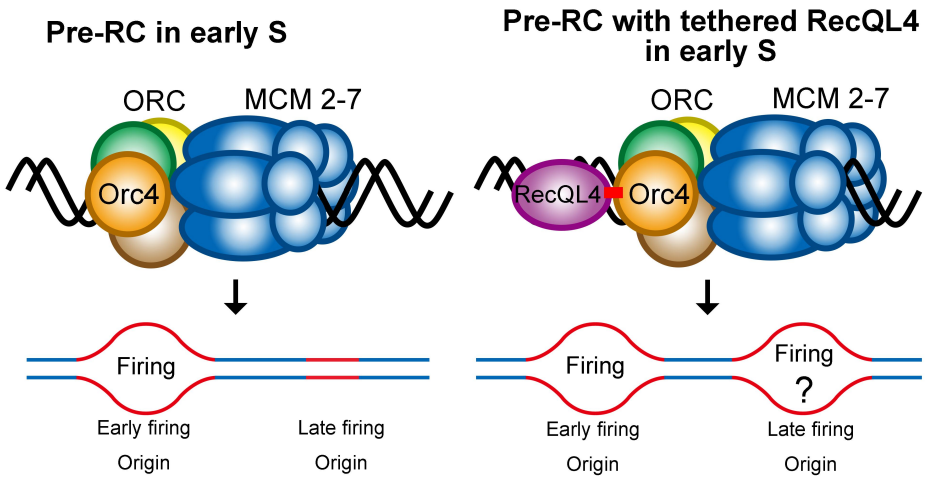
**(A)** Time schedule for cell synchronization. **(B)** HeLa cells were synchronized at the G1/S boundary and released for 1 h (early S phase) or 6 h (late S phase). Cells were harvested, and ChIP assays were performed using the described antibodies at the indicated times. Relative binding of proteins to the β-globin origin compared to the non-origin region (-21 kb distal region from the β-globin origin) was examined by quantitative PCR analysis. The mean values ± standard deviation with p-values are shown. 'ns' denotes 'not significant'. ***$p < 0.001$.

## 4.2. Tethering RecQL4, Mcm10, or And-1 on the pre-RC is sufficient to activate late replicating origins in early S phase

Since RecQL4, Mcm10, and And-1 proteins are recruited to the replication origin at the time of its activation, I tested whether their aberrant binding to replication origins affected the timing of origin activation during S phase (**Figure 2A**). RecQL4 proteins fused to the N-terminus of ORC components were expressed in HeLa cells to force tethering of RecQL4 proteins on the pre-RC, and then the nascent DNA synthesis at early or late replicating origins was determined by bromodeoxyuridine-immunoprecipitation (BrdU-IP) followed by quantitative PCR analysis (**Figure 2B**).

In HeLa cells, nascent DNA synthesis at the lamin B2 origin, a typical early replicating origin in human cells (Abdurashidova et al., 2000; Todorovic et al., 2005), was dominant in early S phase, while replication at late replicating origins such as GRM8 (Cadoret et al., 2008) and β-globin origins was almost negligible during early S phase and occurred mostly in late S phase. To validate this findings, BrdU IP qPCR was performed. I confirmed that the lamin B2 origin was replicated in early S. The GRM8 and the β-globin origin were activated in late S phase (**Figure 3 and Figure 5C**).

A

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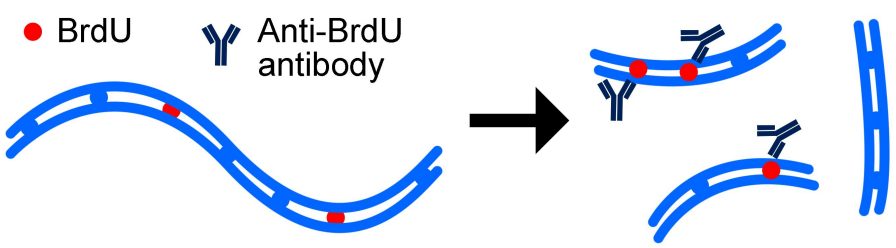



**Figure 2. An experimental diagram for verifying initial DNA synthesis.**

**(A)** (left panel) Pre-RCs are formed onto every replication origins but only early firing origins are activated in early S. (right panel) Late firing origin with pre-RCs bearing an aberrant RecQL4 could be activated in early S phase. **(B)** An diagram of BrdU-IP qPCR experiment. (left panel) BrdU is incorporated into genomic DNA. (right panel) DNA is sheared, heated and cooled for exposure of BrdU epitope. The pull-down DNA is analyzed by qPCR.

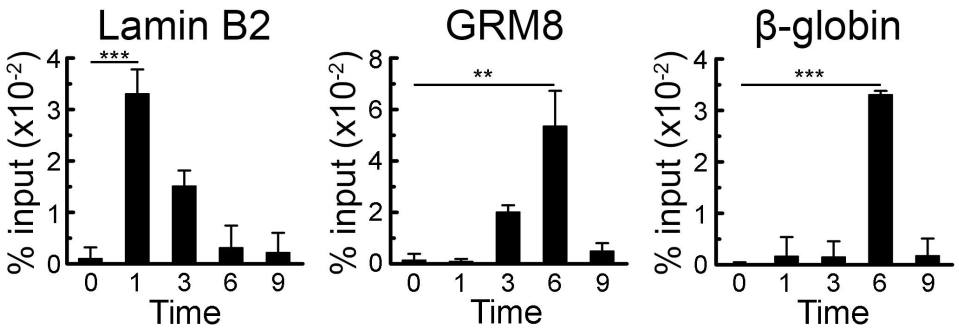


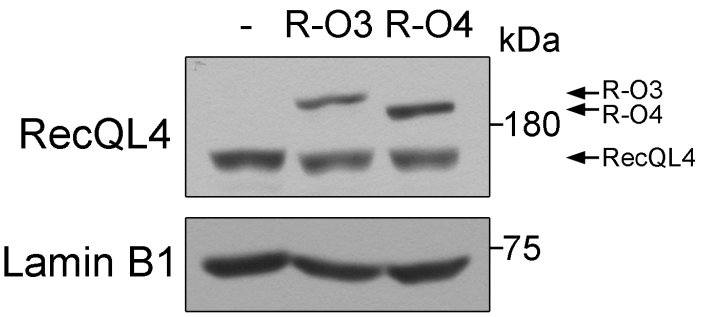

**Figure 3. Replication timing of origins in HeLa cells.**

Cells were synchronized at G1/S and released for the indicated time (h). Nascent DNA synthesis was examined by BrdU-IP. p values: $^{**}p < 0.01$; $^{***}p < 0.001$.

The eukaryotic origin recognition complex (ORC) is composed of 6 subunits: Orc1, Orc2, Orc3, Orc4, Orc5, and Orc6. ORC is ring-like in shape. ORC binds to and encircles the DNA to form the pre-RC. Orc1 and Orc2 serve as a "gate" for the ORC to enclose the DNA. Orc6 is the smallest of the Orc subunits while Orc3 and Orc4, located opposite to each other, are moderately sized (Bleichert et al., 2015). To force binding of RecQL4 with the pre-RC, the RecQL4 protein was fused with either Orc3 or Orc4. Unlike RecQL4-Orc3, the expression of the RecQL4-Orc4 fusion protein **(Figure 4A)** increased the β-globin origin binding of RecQL4 in early S phase **(Figure 4B)** suggesting that the interaction between RecQL4 and Orc4 is necessary for RecQL4 origin binding on the pre-RC.

Expression of RecQL4-Orc4 fusion proteins in HeLa cells (**Figure 5A**) increased nascent DNA synthesis at the late replicating origins **(Figure 5B**, GRM8 and β-globin origins) in early S phase. The level of nascent DNA synthesis at the β-globin origin observed in these cells was comparable to the level of DNA synthesis observed in the late S phase in HeLa cells (**Figure 3**).

A

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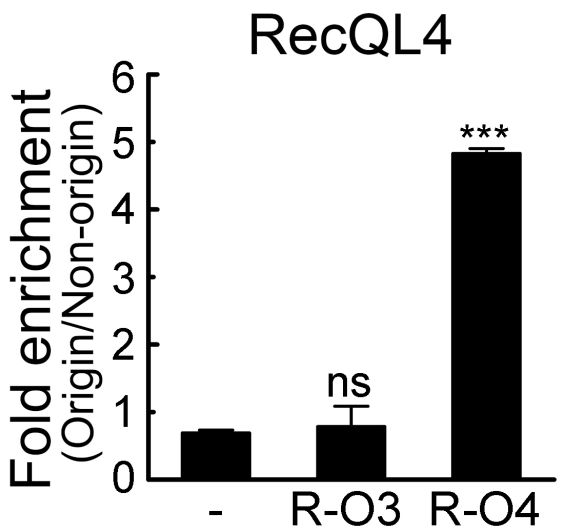



**Figure 4. Determination of RecQL4 binding site onto ORC.** HeLa cells transfected with RecQL4-Orc3 (R-O3) or RecQL4-Orc4 (R-O4) were synchronized at early S phase. **(A)** Expression of fusion proteins. **(B)** Origin binding of RecQL4 was determined by ChIP. - : no transfection. p values: ns, not significant; ***$p < 0.001$.

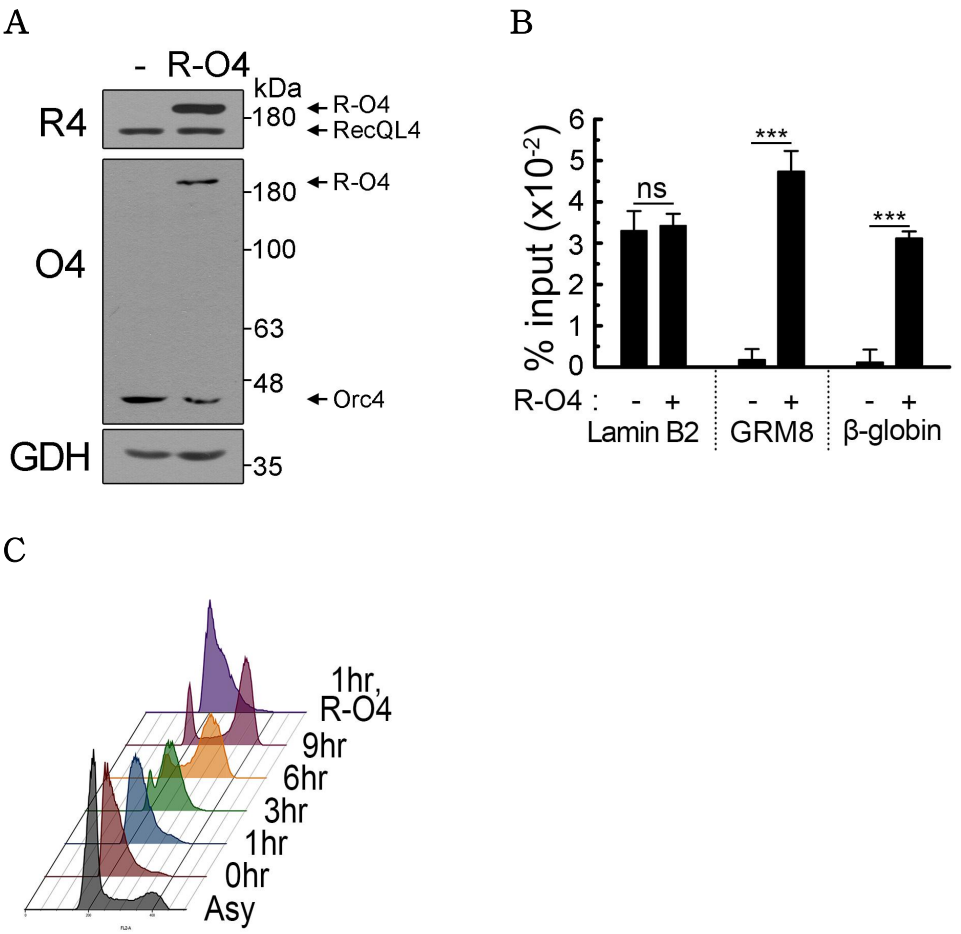



**Figure 5. RecQL4-Orc4 triggers origin activation of late firing origins in early S phase.**

HeLa cells transfected with RecQL4-Orc4 (R-O4) were synchronized at early S phase. (**A**) Expression of RecQL4-Orc4 fusion proteins. R4, RecQL4; O4, Orc4; GDH, GAPDH. (**B**) RecQL4 tethered on the pre-RC induces origin activation. Cells transfected with R-O4 were synchronized at early S phase, and nascent DNA synthesis was determined by BrdU-IP. (**C**) The cell cycle profile of cells prepared in (**A**), (**B**) and **Figure 3**.
p values: ns, not significant; ***$p < 0.001$.

To investigate whether CDK and DDK, two major kinases regulating origin activation, contribute to the origin activation induced by the tethered RecQL4, R-O4 expressing cells were treated with inhibitors for CDK2 (Nu6140) or DDK (PHA-767491) followed by BrdU-IP analysis at early S phase. The nascent DNA synthesis depended on two S phase promoting kinases, CDK and DDK (**Figure 6**), similar to the nascent DNA synthesis observed during normal DNA replication. Therefore, increases in nascent DNA synthesis observed in these cells appeared to be caused by early activation of the late replicating origins.

The origin activation by aberrant binding of RecQL4 could be an effect from unequal DNA transfection. To exclude this possibility, I created a stable cell line that expresses RecQL4-Orc4. Increases in nascent DNA synthesis at the late replicating origins in early S phase were also observed in the cell line stably expressing RecQL4-Orc4 proteins from the Tet-inducible promoter (**Figure 7A and B**). Cell cycle profiles, meawhile, were almost unaffected by RecQL4-Orc4 expression (**Figure 7C**).

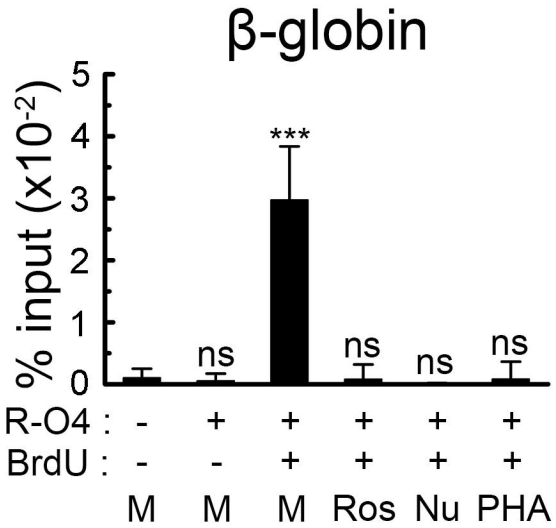



**Figure 6. Origin activation by tethered RecQL4 depends on both DDK and CDK.**

Synchronized Hela cells transfected with R-O4 were treated with 10 mM Roscovitine (Ros), Nu6140 (Nu), or PHA-767491 (PHA) for 1 h at early S phase, and nascent DNA synthesis was examined. M, Mock treatment. p values: ns, not significant; ***$p < 0.001$.

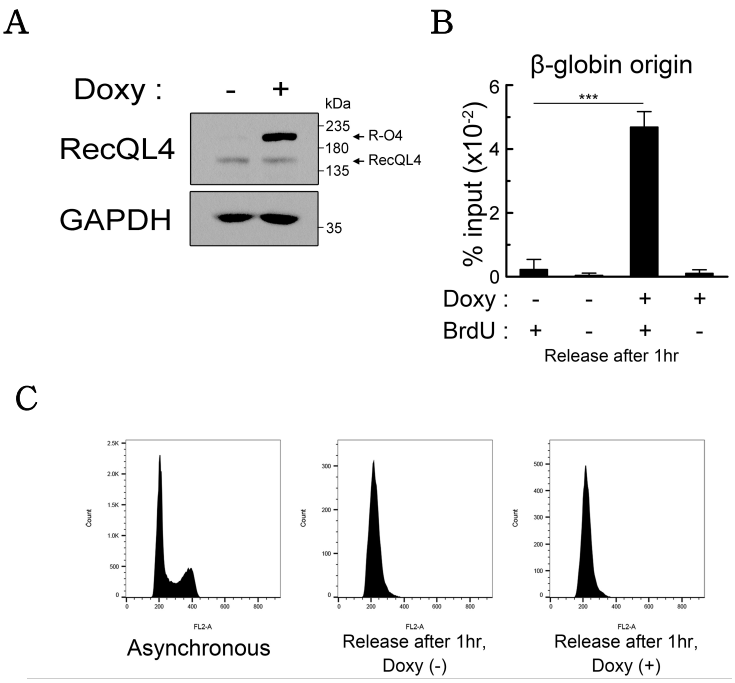


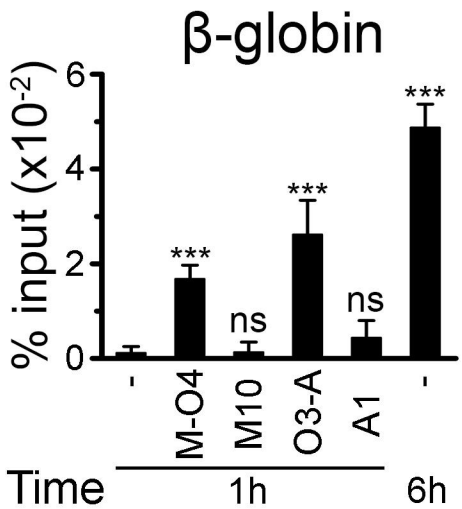

**Figure 7. The β-globin origin is activated at early S phase in HeLa cells stably expressing RecQL4-Orc4 proteins.**

HeLa-RO4 cells stably expressing RecQL4-Orc4 proteins from the deoxycycline-inducible promoter were treated with 2mM thymidine for 18 h and released into the cell cycle in fresh medium for 2 h. Next, doxycycline (1 μg/mL) was added to express RecQL4-Orc4 proteins. After incubation for 10 h, thymidine was added at 2 mM and further incubated for 13 h to arrest the cell cycle at the G1/S phase. Cells were released to early S phase in fresh medium for 1 h, and the expression of RecQL4-Orc4 proteins (arrowhead) in these cells (A) and nascent DNA synthesis at the β-globin origin (B) were examined by Western blotting and BrdU-IP, respectively. ***$p < 0.001$. The cell cycle profiles of these cells (C) were determined by flow cytometry analysis after staining with propidium iodide.

As RecQL4 proteins loaded onto replication origins interact with Mcm10 and And-1 proteins, and all these proteins are required for assembly of the CMG complex and origin activation in human cells (J. S. Im et al., 2015), I tested whether early activation of the late replicating origins also occurred by tethering Mcm10 or And-1 on the pre-RC. Although the levels of nascent DNA synthesis were different, tethering Mcm10 or And-1 on the pre-RC by expressing Mcm10-Orc4 or Orc3-And-1 fusion proteins significantly increased nascent DNA synthesis at the β-globin locus in early S phase (**Figure 8**), suggesting that tethered Mcm10 or And-1 proteins also induced activation of late replicating origins in early S phase. Therefore, loading of factors required for CMG complex assembly on the replication origins seems to be sufficient to induce activation of replication origins during S phase.

Any one of RecQL4, Mcm10 and And-1 tethered on the pre-RC may recruit two other proteins functioning together for CMG assembly and origin activation. To confirm this notion, the early activation of late firing origin was observed in the condition with or without those of proteins. RecQL4 tethering on the pre-RC did not force early activation of the late replicating origin if Mcm10 or And-1 was depleted by siRNA (**Figure 9**). Thus, unscheduled activation of late firing origins by RecQL4 tethering requires Mcm10 and And-1.

A

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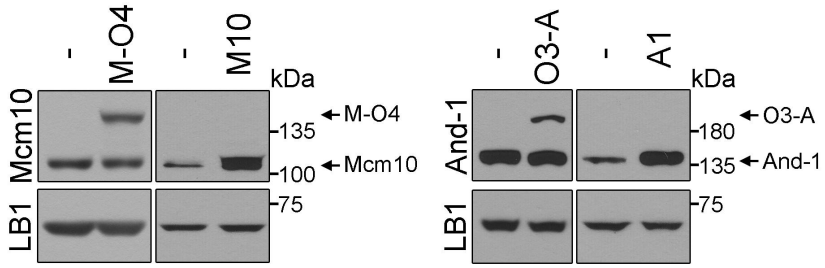



**Figure 8. Tethering Mcm10 or And-1 on the pre-RC triggers early activation of the β-globin origin.**

(**A**) Nascent DNA synthesis at the β-globin origin was examined in cells expressing Mcm10-Orc4 (M-O4), Mcm10 (M10), Orc3-And-1 (O3-A), or And-1 (A1) at early S phase (1 h). (**B and C**) (**B**) and (**C**) show expression levels of these proteins, and arrowheads indicated M-O4 and O3-A proteins. LB1, Lamin B1. p values: ns, not significant; **p < 0.01; ***p < 0.001.

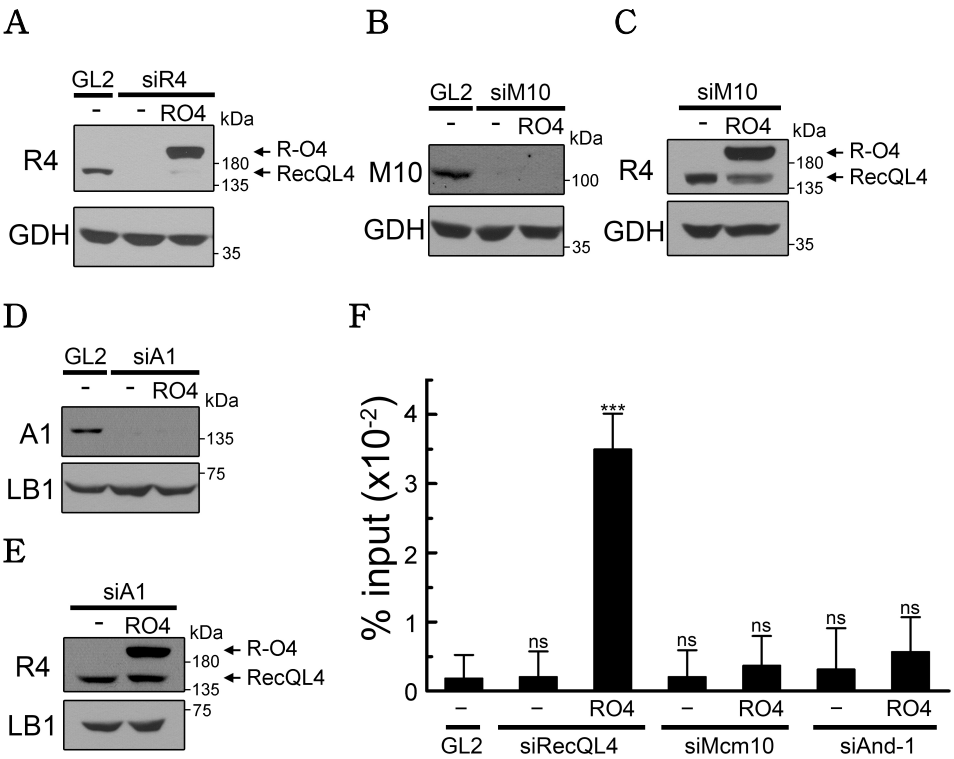



**Figure 9. Early activation of late replicating origins induced by RecQL4 tethering on the pre-RC requires both Mcm10 and And-1.**

HeLa cells depleted of indicated proteins were transfected with plasmids expressing RecQL4-Orc4 (RO4) and synchronized at early S phase by a double thymidine block and release. Nascent DNA synthesis at the β-globin origin (**F**) and cellular levels of the indicated proteins (**A-E**) were examined by BrdU-IP and Western blotting analyses. R4, RecQL4; M10, Mcm10; A1, And-1; LB1, Lamin B1; GDH, GAPDH. p values: ns, not significant; ***$p < 0.001$.

## 4.3. Recruitment of Cdc45 to replication origins by tethered RecQL4 on the pre-RC does not depend on CDK activity

In mammalian cells, concerted action of two S phase promoting kinases, CDK and DDK, is required for recruitment of replication initiation factors on the replication origins and their action. I examined whether recruitment of replication initiation factors forced by the tethered RecQL4 depended on these two kinase activities. When I examined the amount of replication initiation proteins on the late replicating origin in early S phase by *in vivo* crosslinking followed by ChIP analyses, expression of RecQL4-Orc4 proteins clearly increased binding of factors required for CMG assembly (Mcm10 and And-1) as well as CMG component proteins (Cdc45 and Sld5), as expected (**Figure 10**). Treatment of the DDK inhibitor (PHA767491) in these cells reduced origin binding of Mcm10, And-1, Cdc45, and Sld5, suggesting that binding of Mcm10, And-1, Cdc45, and the GINS complex forced by the tethered RecQL4 on the pre-RC depended on DDK activity (**Figure 10**). The binding of Mcm10, And-1, and Sld5 also depended on CDK activity, which was judged by the decrease in origin binding after CDK inhibitor (NU6140) treatment. Conversely, origin binding of Cdc45 was still observed in cells treated with CDK inhibitor, suggesting that origin binding of Cdc45 forced by tethered RecQL4 on the pre-RC did not depend on CDK activity. Furthermore,

CDK-independent recruitment of Cdc45 on replication origins forced by the tethered RecQL4 could occur in the absence of recruitment of other essential initiation factors such as Mcm10, And-1, and Sld5 (**Figure 10**), suggesting that RecQL4 may play its role by directly recruiting either Cdc45 or factors required for Cdc45 recruitment, such as Treslin.

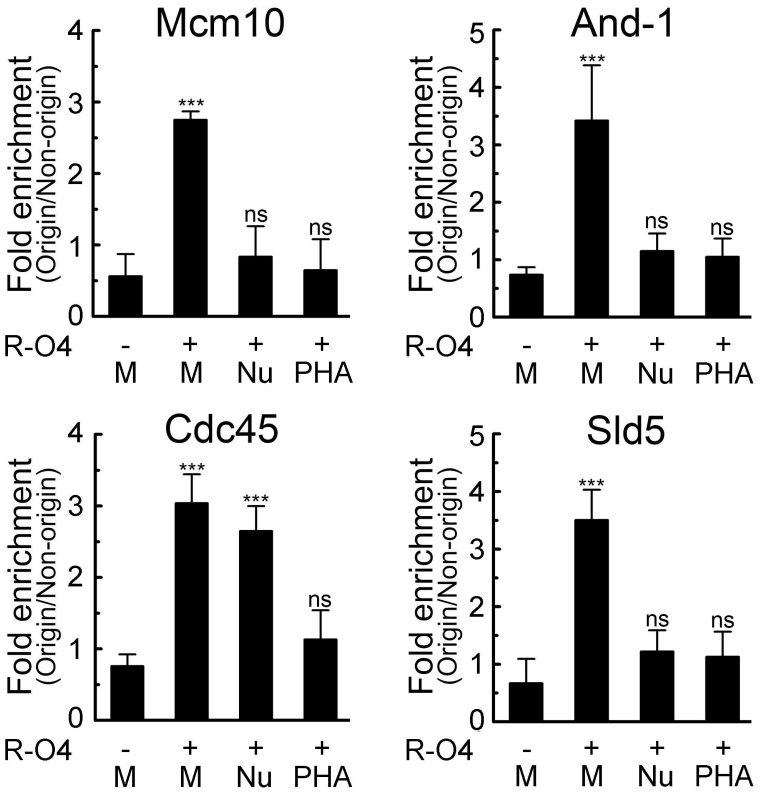



**Figure 10. Influence of CDK and DDK on origin association of replication proteins induced by RecQL4 tethering on the pre-RC.**

HeLa cells expressing RecQL4-Orc4 (R-O4) were synchronized at G1/S and released to S phase in the presence of 10 μM Roscovitine (Ros), Nu6140 (Nu), or PHA-767491 (PHA). After 1 h, ChIP assays for the β-globin origin were carried out using antibodies targeting Mcm10, And-1, Cdc45, or Sld5. Lanes: -, Mock transfection; M, Mock treatment. p values: ns, not significant; ***$p < 0.001$.

## 4.4. CDK phosphorylation of N-terminal Sld2 homology domain in RecQL4 protein plays essential role for activation of replication origins

While CDK phosphorylation of replication factors is essential to initiate DNA replication, the exact role of individual protein phosphorylation during the initiation processes is not yet elucidated in human cells. Therefore, I decided to determine the role of RecQL4 phosphorylation by CDK during the initiation process using this tethering system. Since there are many candidates for essential CDK phosphorylation sites in RecQL4 proteins, I first determined the minimal region of RecQL4 proteins to force early activation of late replicating origins by tethering onto the pre-RC. As shown in **Figure 11A-D**, overexpression of RecQL4 alone or tethering N-terminus deleted fragment (ND1) on the pre-RC did not induce β-globin origin activation. On the other hand, tethering N-terminal fragments, CD1 (amino acids 1-241) or CD2 (amino acids 1- 427) on the pre-RC forced early activation of β-globin origins, although the level of nascent DNA synthesis was somewhat lower than the level of synthesis caused by tethering of full length RecQL4 proteins (**Figure 11A-D**). This result suggests that the N-terminus of the RecQL4 protein (CD1), including the Sld2 homology domain, contains minimal regions required for initiation of DNA replication and origin localization of RecQL4 than its amount is important for origin activation.

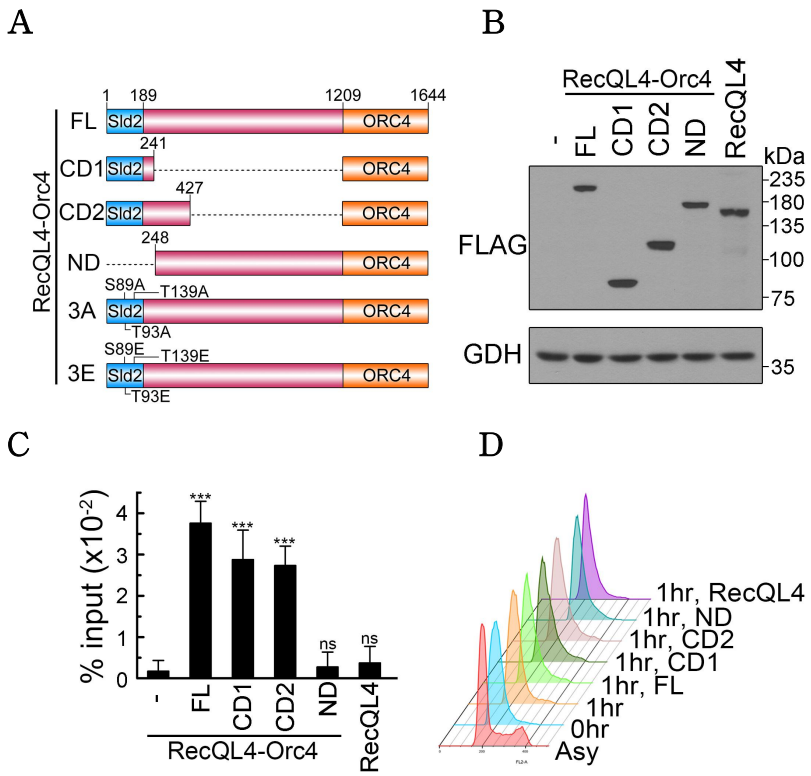



**Figure 11. N-terminus and origin localization of RecQL4 are essential for origin activation.**

(**A**) Schematic diagrams of RecQL4-Orc4 variants. All RecQL4 variants contained 2FLAG tags at the N-terminus. Sld2, a yeast Sld2 homology domain; FL, full-length; CD, C-terminal deleted; ND, N-terminal deleted; 3A, alanine substitutions of three CDK phosphorylation sites (S89, T93, and T139); 3E, glutamic acid substitutions of three CDK phosphorylation sites. (**B-D**) HeLa cells were transfected with plasmids for indicated RecQL4 variants-Orc4 and synchronized at early S phase. The level of expressed RecQL4 variants was determined by Western blotting (**B**), and nascent DNA synthesis at the β-globin origin was examined by BrdU-IP (**C**). The cell cycle profiles of these cells were shown by flow cytometry analysis (**D**).

To determine the role of CDK phosphorylation, I created alanine substitution mutants of all possible CDK phosphorylation sites in the CD1 region and determined their activity to support early activation of late replicating origins by tethering on the pre-RC. When serine or threonine at any CDK phosphorylation site in the CD1 region was individually substituted with alanine, early activation of the β-globin origins was still observed by tethering these mutant proteins (S89A, T93A, and T139A) on the pre-RC, although the levels of nascent DNA synthesis were relatively low (**Figure 12A**). In contrast, alanine substitution of all three phosphorylation sites (3A) almost completely abolished the ability to induce early activation of the β-globin origins. Further, the phospho-mimetic mutant with glutamic acid substitution of all phosphorylation sites (3E) showed the ability to support early activation of the β-globin origins (**Figure 12A**). These results suggested that CDK phosphorylation of all three sites in CD1 plays important roles to activate the replication origins. To confirm the role of these three phosphorylation sites in the context of the full-length RecQL4 protein, I constructed the phospho-deficient (3A) or phospho-mimetic (3E) mutant of the full-length RecQL4 protein containing alanine or glutamate substitution of all three CDK phosphorylation sites in the CD1 region and examined its ability to induce early activation of late replicating origins by tethering onto the pre-RC. Tethering 3A mutant of the RecQL4 protein on the pre-RC barely induced activation of the β-globin origins,

while tethering 3E mutant of the RecQL4 protein on the pre-RC resulted in a significant increase in β-globin origin activation, which is comparable to tethering of the wild-type RecQL4 protein (**Figure 12B**). Collectively, these results suggested that CDK phosphorylation of the three phosphorylation sites in the Sld2 homology domain of RecQL4 proteins plays essential roles for activation of replication origins in human cells.

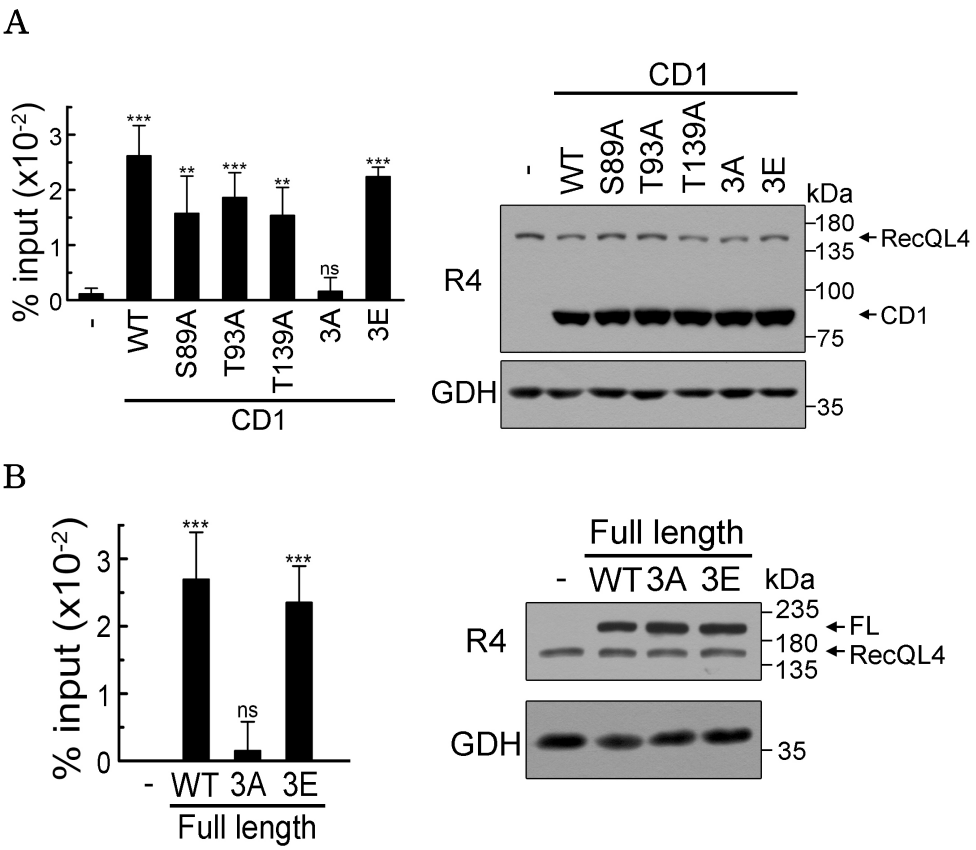



**Figure 12. CDK phosphorylation of RecQL4 N-terminus is essential for origin activation.**

(**A and B**) All three phosphorylation sites at the N-terminus of RecQL4 are important for origin activation. HeLa cells were transfected with plasmids for various RecQL4 CD1-Orc4 (**A**) or full-length RecQL4-Orc4 (**B**) containing the indicated mutations and synchronized at early S phase. Nascent DNA synthesis at the β-globin origin (left panel) and expression levels of individual proteins (right panel) were examined by BrdU-IP and Western blotting, respectively. p values: ns, not significant; **p<0.01; ***p < 0.001.

## 4.5. CDK phosphorylation of RecQL4 N-terminal domain is required for And-1 and GINS recruitment onto replication origin

To explore the exact role of RecQL4 N-terminal phosphorylation by CDK for origin activation, the recruitment of replication initiation factors on the late replicating origin in early S phase was examined after tethering phospho-deficient (3A) or phospho-mimetic (3E) mutants of full-length RecQL4 proteins on the pre-RC. As expected, Cdc45 recruitment onto the replication origin, which did not depend on CDK activity as shown in **Figure 10**, occurred by tethering phospho-deficient, phospho-mimetic, or wild-type RecQL4 proteins (**Figure 13A**). The recruitment of Mcm10, which was shown to be CDK dependent (**Figure 10**), was still observed by tethering phospho-deficient RecQL4 proteins (**Figure 13**). On the other hand, recruitment of And-1 or Sld5, which also depended on CDK activity (**Figure 10**), was not observed by tethering phospho-deficient mutant RecQL4 proteins (**Figure 13**). These results suggested that CDK phosphorylation of the RecQL4 N-terminus is essential for recruitment of the And-1 and GINS complex, but not for Mcm10.

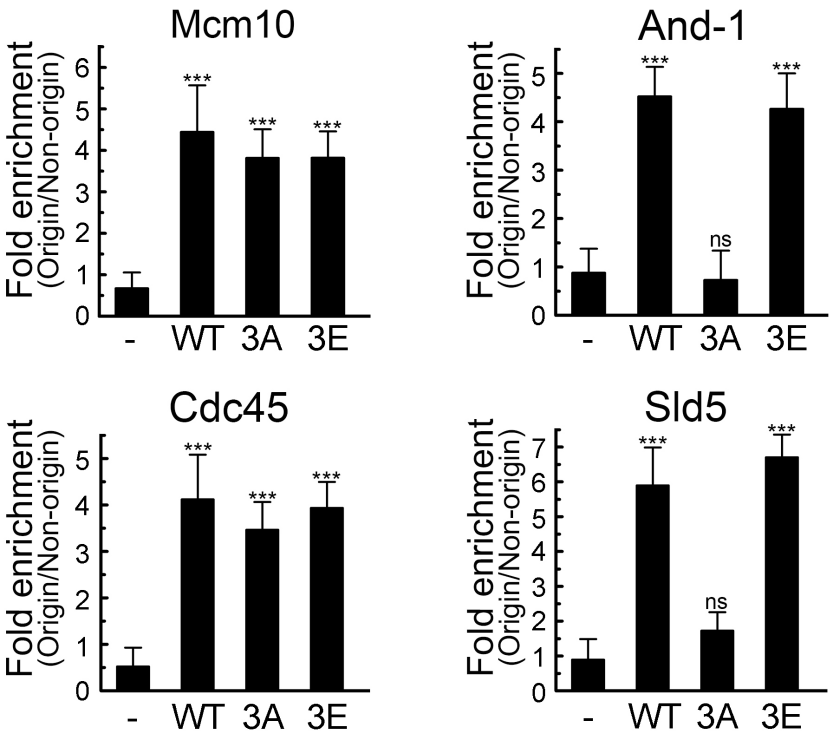



**Figure 13. CDK phosphorylation of RecQL4 N-terminus is required for origin association of And-1 and Sld5.**

Origin association of replication proteins induced by tethered RecQL4 with mutations in CDK phosphorylation sites. HeLa cells transfected with wild-type (WT), phospho-deficient (3A), or phospho-mimetic (3E) forms of full-length RecQL4-Orc4 were synchronized at early S phase. Then, the association of replication proteins on the β-globin origin was analyzed by ChIP. p values: ns, not significant; ***$p < 0.001$.

Because CDK phosphorylation of the RecQL4 N-terminus is essential for recruitment of the And-1 and GINS complex, but not for Mcm10, I tested whether the CDK phosphorylation affects protein interaction between RecQL4 and each protein. Co-immunoprecipitation (IP) analysis showed that the phospho-deficient form of RecQL4 stably interacted with Mcm10, but not with And-1 or Sld5; in contrast, wild-type RecQL4 stably interacted with And-1 and Sld5 as well as Mcm10 in HeLa cells (**Figure 14 A-C**). Therefore, CDK phosphorylation of the RecQL4 N-terminus was not required for RecLQ4 interaction with Mcm10, but appeared to play its role in the recruitment of And-1 and Sld5 on replication origins by increasing the interaction of RecQL4 with And-1 and Sld5.

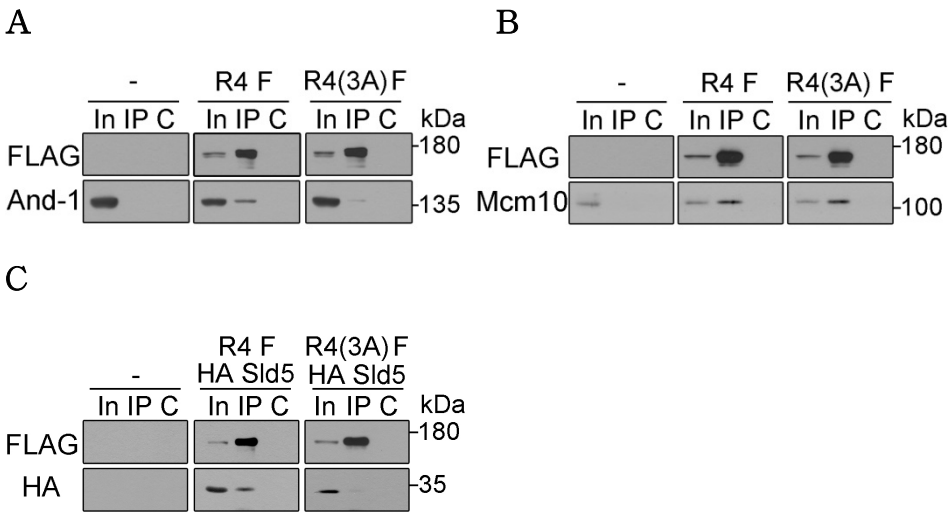



**Figure 14. CDK phosphorylation of RecQL4 N-terminus is required for protein interaction of And-1 and Sld5, not for Mcm10.**

(**A-C**) Physical interaction of wild-type or phospho-deficient RecQL4 with And-1, Sld5, or Mcm10. HeLa cells were transfected with FLAG-tagged wild-type (R4 F) or phospho-deficient (3A) RecQL4 (**A and B**), or transfected with indicated RecQL4 and HA-tagged Sld5 (**C**). Cells were synchronized at early S phase, and the interaction between indicated proteins was analyzed by co-IP with anti-FLAG antibodies. Lanes: In, 5% of input for IP; C, control IP in the presence of FLAG peptides (0.2 mg/mL).

However, the absence of Sld5 recruitment could be indirectly caused by lack of And-1 recruitment because And-1 has been shown to interact with GINS and is required for CMG complex assembly (J.-S. Im et al., 2009; Moiseeva et al., 2017). To test this possibility, I tethered RecQL4 proteins on the pre-RC in the absence of And-1 and examined Sld5 recruitment onto the replication origin. As shown in **Figure 15A and B**, the recruitment of Sld5 proteins onto β-globin origins still occurred in the absence of And-1 by tethering wild-type or phospho-mimetic RecQL4 proteins, although replication at the β-globin origin was not induced by tethering in the absence of And-1 proteins (**Figure 15C and D**). Therefore, phosphorylation of the RecQL4 N-terminus by CDK appears to be responsible for the interaction and recruitment of both And-1 and the GINS complex.

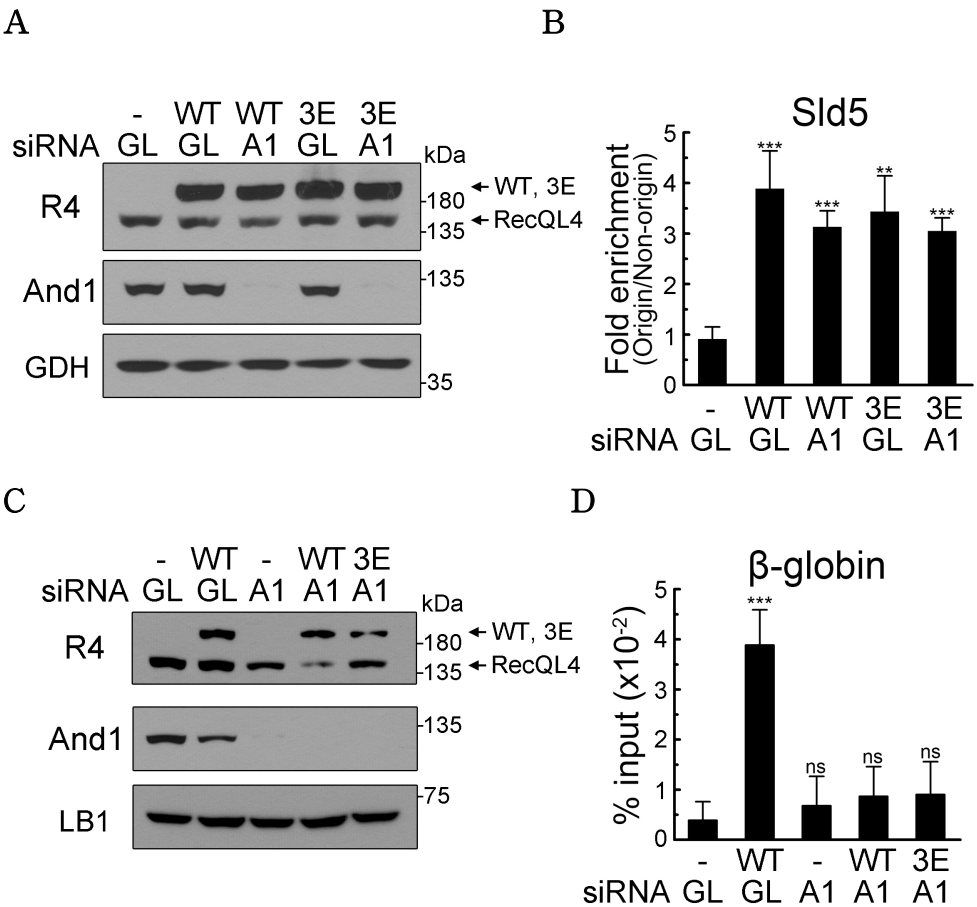



**Figure 15. CDK phosphorylation of RecQL4 is more important for origin binding of Sld5.**

Mock-depleted (GL) or And-1 (A1) depleted HeLa cells were transfected with wild-type (WT) or phospho-mimetic (3E) RecQL4-Orc4 (**A and C**) and synchronized at early S phase. Then, the association of Sld5 proteins with β-globin origins (**B**) and nascent DNA synthesis at β-globin origins (**D**) were determined by ChIP and BrdU-IP analyses, respectively. p values; ns, not significant; **p < 0.01; ***p < 0.001.

## 4.6. Perturbation of replication timing control programs by forced activation of late replicating origins induces replication stress that is relieved by inhibiting transcription

Since RecQL4 tethering on the pre-RC induces unscheduled activation of late replicating origins, I investigated how cells responded to the perturbation of replication timing control. At first, I examined whether replication stress was induced by activation of late replicating origins in early S phase. In U2OS cells stably expressing RecQL4-Orc4 proteins from the Tet-inducible promoter, increases in phosphorylated ATR and phosphorylated RPA, a well-known target of ATR kinase (Liu et al., 2012; Vassin et al., 2009), were observed after addition of doxycycline (**Figure 16**), suggesting that the unscheduled activation of late replicating origins induced replication stress, which resulted in increased single stranded DNAs and activation of ATR, a master kinase for the replication checkpoint response. Immunostaining of RPA proteins in these cells clearly showed accumulation of single stranded DNAs on chromatins after induction of RecQL4-Orc4 proteins (**Figure 17, 18B and C**). However, phosphorylated histone H2AX (γH2AX), a marker of DNA double strand breaks, was not significantly increased during this condition (**Figure 16**), indicating that stress induced by forced activation of late replicating origins can be overcome by cellular responses to replication stress, such as the ATR pathway.

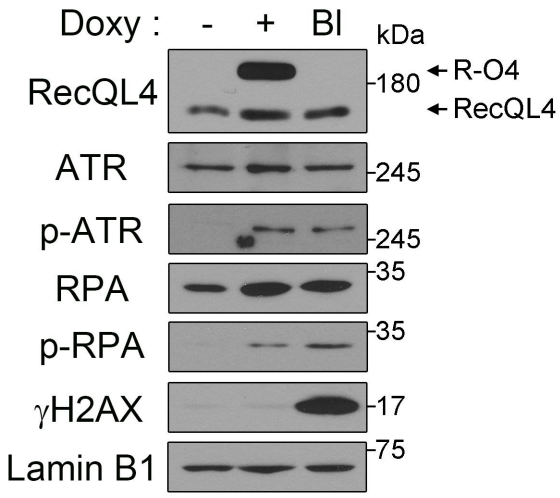



**Figure 16. Unscheduled activation of replication origins induces replication stress.**

U2OS cells stably expressing RecQL4-Orc4 proteins from the doxycycline-inducible promoter (U2OS-RO4) were cultured in the absence (-) or presence (+) of 3μg/ml doxycycline for 48 h. Then, the levels of p-ATR (S428), p-RPA (S33), and γH2AX were examined by Western blotting. As a positive control, cells were treated with 30 μg/ml bleomycin (Bl), a drug for induction of DNA double strand breaks, strand for 2 h.

Over-replication induced by expression of limiting replication factors or oncogene activation has been shown to increase replication stress by depletion of dNTP pools in some cells (Bester et al., 2011; Kohler et al., 2016). However, RPA foci were still observed after addition of nucleosides (**Figure 17**), suggesting that depletion of dNTPs was not the main reason for the accumulation of single stranded DNAs in cells expressing RecQL4-Orc4.

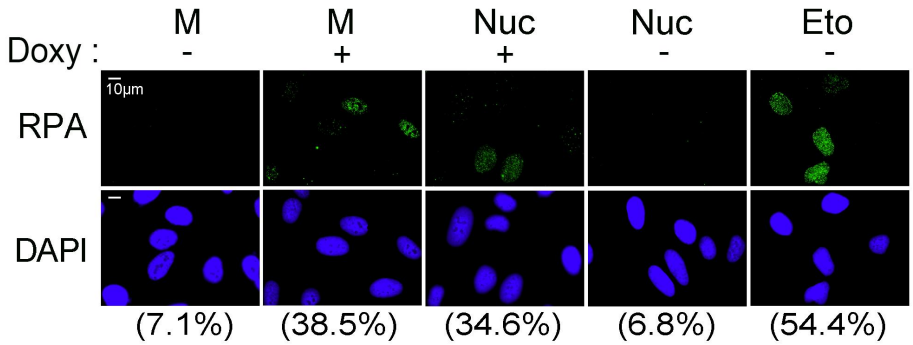
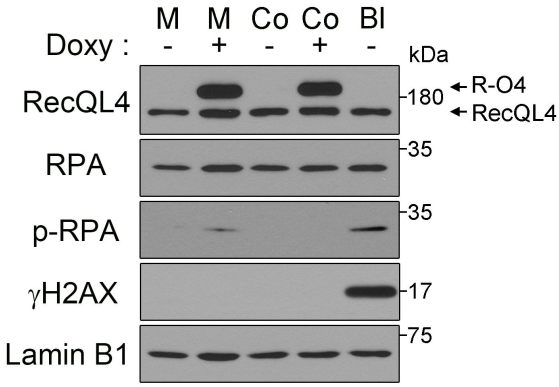



**Figure 17. Nucleosides addition does not relieve replication stress from RecQL4-Orc4.**

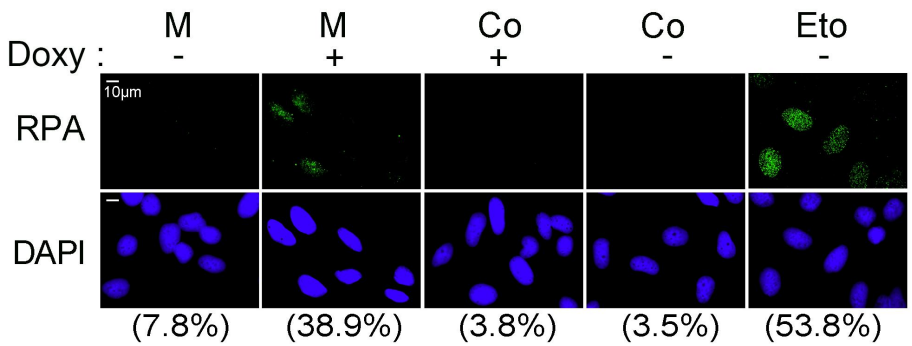
U2OS-RO4 cells were cultured in the absence (-) or presence (+) of 3 μg/ml doxycycline and 50 μM nucleosides (Nuc) for 48 h. RPA foci formation was examined by staining with anti-RPA32 antibody. M, Mock treatment. The numbers at the bottom of each image indicate the percentage of RPA foci positive cells.

To test whether transcription-replication conflicts are the cause of replication stress by RecQL4-Orc4, U2OS-RO4 cells are incubated with or without transcription inhibitors. The treatment of transcription inhibitors such as cordycepin or α-amanitin almost completely reduced RPA phosphorylation (**Figure 18A**) and RPA foci (**Figure 18B and C**). Therefore, unscheduled activation of replication origins in cells expressing RecQL4-Orc4 proteins seemed to induce replication stress by increasing transcription-replication conflicts.

A




B




C

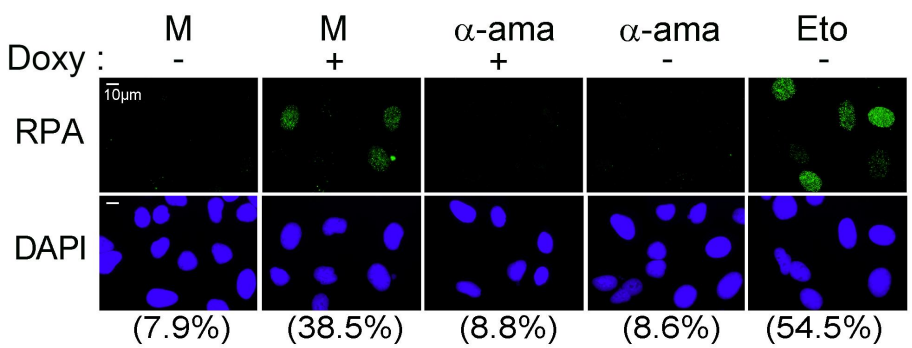



Figure 18. Transcription inhibition reduces replication stress by RecQL4-Orc4.

U2OS-RO4 cells were grown in the absence (-) or presence (+) of 3 μg/ml doxycycline for 44 h. Cells were treated with 75μM cordycepin (Co) or 40 μg/ml α-amanitin (α-ama) for 4 h. The level of indicated proteins was examined by Western blotting (**A**) and ssDNA accumulation was analyzed by staining with anti-RPA32 antibody (**B and C**). As a positive control, cells were treated with 30 μg/ml bleomycin (Bl) for 2 h or 10 μM etoposide (Eto), DNA topoisomerase II inhibitor, for 1 h. M, Mock treatment. The numbers at the bottom of each image indicate the percentage of RPA foci positive cells.

# Ⅴ. Discussion

In this study, RecQL4 was tethered on replication origins by expressing RecQL4-Orc4 fusion proteins, and I found that the tethered RecQL4 protein on the pre-RC induced recruitment of replication initiation factors, assembly of the CMG complex, and nascent DNA synthesis during S phase in human cells. Since overexpression of RecQL4 alone did not induce late origin activation (**Figure 11B-D**), association of RecQL4 with replication origins rather than increase in RecQL4 protein level appeared to be critical for forced activation of late replicating origins. The process of late origin activation in early S phase by RecQL4 tethering was summarized in **Figure 19**.

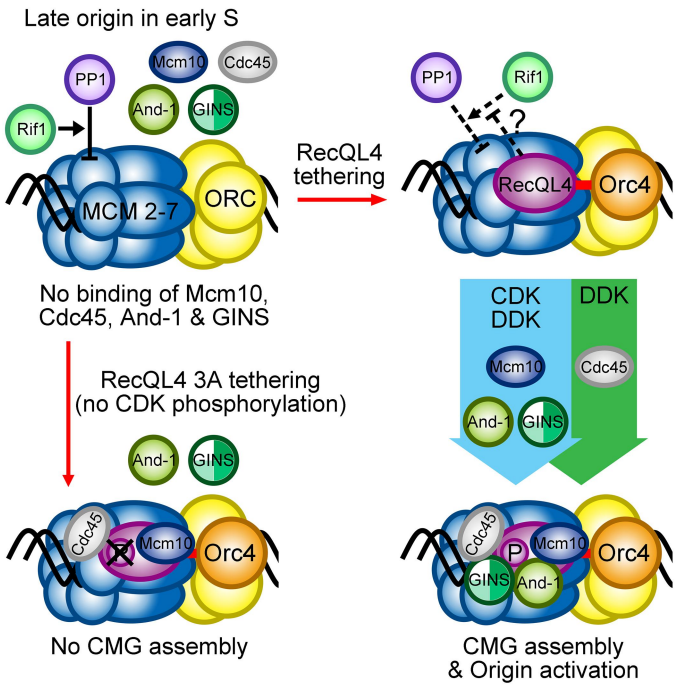



**Figure 19. Diagram showing the forced activation of late replicating origins by RecQL4 tethering.**

In early S phase, late origin activation is prevented by Rif1 dependent PP1 action. Tethered RecQL4 somehow overcomes PP1 action and induces recruitment of other initiation factors in a manner dependent upon DDK (for Cdc45) or DDK and CDK (for Mcm10, And-1, and GINS). This leads to CMG assembly and late origin activation in early S phase. Tethering of phospho-deficient RecQL4 defective in CDK phosphorylation failed to induce origin activation due to lack of origin binding of And-1 and GINS.

## 5.1. An forced origin activation by RecQL4-Orc4 appears to follow the conserved mechanism of origin activation

While the timing of origin activation in mammalian cells appears to be governed by characteristics of the replication origins and replication timing control program during S phase (Fu et al., 2018), tethered RecQL4 on the late replicating origin somehow overcame those controls to activate late replicating origins in early S phase. RecQL4 was shown to be required for origin binding of Mcm10 and And-1, and co-dependent binding of RecQL4 and Mcm10 on replication origins is an early event that occurs before the recruitment of And-1 and the CMG assembly (J.-S. Im et al., 2009; J. S. Im et al., 2015). All known initiation factors tested in this study were recruited to the late replicating origin in early S phase by tethered RecQL4 (**Figure 10**) or RecQL4 N-terminus (**Figure 13**). The nascent DNA synthesis induced by tethered RecQL4 was dependent upon other initiation factors, such as Mcm10 and And-1 (**Figure 9**), and both CDK and DDK activities (**Figure 6**). Since all of these results are consistent with previous observations for the initiation of DNA replication in eukaryotes (J.-S. Im et al., 2009; J. S. Im et al., 2015; Kang et al., 2013; Xu et al., 2009; Zhu et al., 2007), origin activation induced by RecQL4 tethering seems to follow conserved mechanism of origin activation after RecQL4 binding to replication origins.

Therefore, I believe that this system would be useful to determine the role of RecQL4 protein and its modification by CDK in the initiation process of DNA replication in human cells.

## 5.2. Cdc45 recruitment to replication origins by tethered RecQL4 does not depend on CDK activity

In the present study, I was able to determine the mechanism of origin activation in detail using this system. I found that Cdc45 was recruited to replication origins by the tethered RecQL4 even in the absence of CDK activity, while other initiation factors such as Mcm10, And-1, and Sld5 were not (**Figure 10**). This result suggested that the RecQL4 on the pre-RC was directly responsible for the origin association of Cdc45 or Treslin, a factor required for Cdc45 recruitment. Further, the CDK dependency for origin binding of Cdc45 observed in previous studies (J.-S. Im et al., 2009; A. Kumagai et al., 2010, 2011) appeared to be caused by failure of RecQL4 loading onto the replication origin, and the interaction of RecQL4 with Cdc45 or Treslin may not depend on CDK activity. Consistent with this notion, RecQL4 loading onto the replication origin requires both CDK and DDK activities (J. S. Im et al., 2015), and RecQL4 was shown to interact with Cdc45 proteins in cells (Kliszczak et al., 2015; Xu et al., 2009).

## 5.3. RecQL4 N-terminal phosphorylation by CDK is responsible for origin recruitment of And-1 and Sld5, but not for Mcm10

Using this system, I also found that CDK phosphorylation of the RecQL4 N-terminus was essential for origin activation (**Figure 11**) and origin recruitment of And-1 and Sld5, but not for Mcm10 recruitment (**Figure 13**). While origin association of essential initiation factors, except for Cdc45, depended on both CDK and DDK activities (**Figure 10**), the phospho-deficient form of RecQL4 still interacted with Mcm10 (**Figure 14B**) and induced recruitment of Mcm10 on origins, as well as the wild-type or phospho-mimetic form of RecQL4 (**Figure 13**). Therefore, this results clearly showed that phosphorylation of the RecQL4 N-terminus was not required for its interaction with or recruitment of Mcm10 on the replication origins. Phosphorylation of other initiation factors, including Mcm2-7 complex and/or Mcm10 itself, might be responsible for the CDK dependency of Mcm10 recruitment on replication origins. On the other hand, phospho-deficient RecQL4 proteins failed to induce origin recruitment of And-1 and Sld5, and did not show any interaction with And-1 or Sld5 proteins (**Figure 13, Figure 14A and C**). Since the phospho-mimetic form of RecQL4 is capable of recruiting both And-1 and Sld5 on replication origins, and origin association of Sld5 during this condition did not depend on the presence of And-1 proteins (**Figure 15A**

**and B**), CDK phosphorylation of the RecQL4 N-terminus appears to be directly responsible for the recruitment of both And-1 and the GINS complex including Sld5.

## 5.4. RecQL4 plays more roles in replication initiation than Sld2

In yeast cells, DDK phosphorylation of the Mcm2-7 complex is important for recruitment of Cdc45 with Sld3 on replication origins, and no CDK phosphorylation is required for Cdc45 recruitment (Araki, 2016). CDK phosphorylation of several initiation factors, including Sld2 and Sld3, has been shown to facilitate recruitment of a complex that includes GINS and polymerase ε (Heller et al., 2011; Muramatsu et al., 2010). Accordingly, the roles of CDK and DDK in yeast systems appear to be conserved in human cells. Once RecQL4 is loaded onto replication origins, origin binding of Cdc45 depends on DDK activity, but not on that of CDK (**Figure 10**). In addition, CDK phosphorylation of the RecQL4 N-terminus containing the Sld2 homology domain is required for origin binding of the GINS complex, including Sld5 (**Figure 13**). However, RecQL4 is not only required for origin association of the GINS complex as shown in yeast systems, but also required for recruitment of other essential initiation factors, including Mcm10, And-1, and Cdc45. Therefore, RecQL4 in human cells appears to play more diverse roles than yeast Sld2 during the initiation process.

## 5.5. Forced binding of RecQL4 to replication origins affects the replication timing control program

RecQL4 protein tethered on the pre-RC somehow overcomes the replication timing control program and induces early activation of late replicating origins in human cells (**Figure 5**). Eukaryotic cells have multiple replication origins, and the sequential activation of replication origins during S phase is governed by the replication timing control program, which appears to be important to maintain genome integrity (Fu et al., 2018). In yeast, *S. cerevisiae*, availability of limiting initiation factors such as Cdc45, Dbf4, Sld2, and Sld3 has been shown to be important for the control of replication timing, and their overexpression induces premature activation of late replicating origins (Tanaka et al., 2011). While Cdc45 was shown to be a limiting initiation factor and its overexpression affected origin activation in human cells (Kohler et al., 2016), replication timing control program in human cells appeared to be more influenced by architectural features of chromosomes, such as cis-elements in chromosomal DNAs, chromatin structure, and trans-acting factors associated with chromatin (Fu et al., 2018). In mammals, replication timing domains that contain multiple replication origins and replicate concomitantly within a short time window highly co-localize with topologically associated domains (Fu et al., 2018). Further, cis-elements influencing origin firing and

chromatin structure and trans-acting factors such as Rif1 were shown to affect the replication timing control during S phase (Fu et al., 2018; Hayano et al., 2012; Yamazaki et al., 2012). Rif1 is a chromatin associated protein modulating organization of chromatin, and has been shown to recruit protein phosphatase 1 (PP1) to prevent DDK phosphorylation of the Mcm2-7 complex. I still do not clearly understand how RecQL4 on the pre-RC overcame the replication timing control program; however, it seems important to note that both RecQL4 and Rif1 can interact with G-quadruplex (G4) structures. G4 structures have been shown to be associated with replication origins (Hänsel-Hertsch et al., 2017), and Rif1 binds to G4 structures and suppresses replication by recruiting PP1 (Alver et al., 2017; Fu et al., 2018; S.-i. Hiraga et al., 2014; S. i. Hiraga et al., 2017; Kanoh et al., 2015; Sukackaite et al., 2017). Since RecQL4 N-terminus has a strong affinity to G4 structures (Keller et al., 2014), RecQL4 may antagonize the activity of Rif1 by competing for G4 binding or resolving G4 structures. It may also be possible that RecQL4 or factors directly recruited by RecQL4 influence chromatin structure to increase accessibility to limiting initiation factors. Consistent with this notion, And-1 was shown to interact with and stabilize Gcn5, a histone acetyltransferase, in human cells (Li et al., 2012), but its involvement in the replication process has yet to be determined.

## 5.6. Perturbation of replication timing control program by RecQL4-Orc4 expression elevates replication stress by inducing transcription-replication conflicts

Sequential activation of replication origins in mammalian cells is precisely programmed, and the perturbation of this program appears to increase genome instability (Blumenfeld et al., 2017). Increased replication by over-expression of limiting replication initiation proteins such as Cdc45 results in more origin firing and increases in γH2AX (Kohler et al., 2016). Oncogene activation was also shown to increase replication initiation and replication stress (Blumenfeld et al., 2017). Since RecQL4 tethering results in more unscheduled origin activation, it may be possible to increase replication stress, including transcription-replication conflicts, as shown in cells with replication stress induced by cyclin-E expression (Jones et al., 2013). Consistent with this notion, RecQL4 tethering on the pre-RC increased the accumulation of single stranded DNAs, and their accumulation was almost completely reversed by inhibition of transcription (**Figure 18**). In eukaryotic cells, several proteins have been shown to be responsible for resolving replication stress caused by transcription-replication conflicts. ATR is a major checkpoint kinase that responds to replication stress, and Mec1, a yeast homolog of ATR, has been shown to play important roles in limiting

transcription-replication conflicts during replication stress (Hamperl and Cimprich, 2016). Active roles of p53 in replication fork processivity and in preventing transcription-replication conflicts have also been reported (Klusmann et al., 2016; Yeo et al., 2016). FancD2, a component of the FA pathway, plays roles in responding to replication stress and in removal of R-loops (DNA:RNA hybrids) (García-Rubio et al., 2015; Schwab et al., 2015). Although it is not clear whether replication stress induced by tethering RecQL4 on the pre-RC is solely caused by transcription-replication conflicts, it would be interesting to see whether the proteins known to be involved in transcription-replication conflicts play any roles in response to this replication stress.

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# Ⅶ. Abstract in Korean

## 국문초록

진핵생물 DNA 복제에서 복제 원점 활성화 기작은 효모를 이용한 연구 체계로 광범위하게 연구되어왔다. RecQL4와 같은 효모의 포유동물 상동 단백질은 보존된 역할을 하는 것처럼 보이지만, 복제 원점 활성화의 상세한 기작과 그것의 조절은 포유동물 세포에서 여전히 잘 알려지지 않았다. RecQL4는 보존된 RecQ family 헬리케이스의 하나로서, 포유동물 세포에서 DNA 복제 개시에 필수적인 역할을 한다. 포유동물 세포에서 복제 원점 활성화에 필요한 단백질의 분자적 상호작용과 기능을 분석하기 위해, RecQL4-Orc4 융합 단백질을 HeLa 세포에서 발현하여 RecQL4를 복제전복합체(pre-RC)에 연결하였고, 후기 복제 원점(late replicating origin)에서 다른 복제 개시 인자들의 복제 원점 모집과 복제 원점 활성화를 조사하였다. RecQL4-Orc4 융합 단백질 발현으로 RecQL4를 복제전복합체에 결합시키면, 후기 복제 원점은 S기(S phase)의 이른 시기에 활성화되었다. 복제전복합체에 결합된 야생형 RecQL4나 RecQL4 아미노 말단 영역은 Mcm10, And-1, Cdc45, GINS와 같은 복제 개시 필수 단백질을 모집하였고, 이른 S기에 후기 복제 원점에서 신생 DNA 합성을 증가시켰다. 또한, RecQL4-Orc4 발현에 의한 후기 복제 원점 활성화는 Mcm10과 And-1을 필요로 하였다. 이러한 복제 원점 활성화 과정에서, RecQL4는 cyclin 의존성 인산화 효소(CDK) 활성에 의존하지 않으면서 Cdc45를 모집하였고, RecQL4 아미노 말단의 CDK 인산화는 RecQL4가 And-1,

GINS와의 상호작용하고 그것들은 복제 원점으로 모집하는데 필수적이었다. 게다가, 복제전복합체와 RecQL4의 결합에 의한 복제 원점의 활성화는 복제 스트레스를 증가시키고 단일 가닥 DNA(ssDNA)를 축적하게 했다. 이것은 전사(transcription)를 억제하였을 때 회복되었다. 종합하면, 복제 원점에 RecQL4가 오는 것은 S기 동안 복제 원점의 복제 시점을 조절하는 데 가장 중요한 단계이다. 또한, RecQL4-Orc4 발현에 의한 계획되지 않은 복제 원점 활성화는 복제 스트레스를 유도되는데, 이것은 전사-복제 충돌이 원인이다. 이 연구는 복제 원점 활성화에서 RecQL4의 중요성과 복제 개시 및 복제 스트레스를 연구하는데 유용한 모델 시스템을 제공한다.

**주요어** : RecQL4, cyclin 의존성 인산화 효소(CDK), DNA 복제 개시, 복제 원점 활성화, 복제 스트레스, 전사-복제 충돌, 복제 시점 조절

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